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Citation for final published version:

De Leo, Luigina, Aeschlimann, Daniel ORCID: <https://orcid.org/0000-0003-0930-7706>, Hadjivassiliou, Marios, Aeschlimann, Pascale, Salce, Nicola, Vatta, Serena, Ziberna, Fabiana, Cozzi, Giorgio, Martelossi, Stefano, Ventura, Alessandro and Not, Tarcisio 2018. Anti-transglutaminase 6 autoantibody development in children with celiac disease correlates with duration of gluten exposure. *Journal of Pediatric Gastroenterology and Nutrition* 66 (1) , pp. 64-68. 10.1097/MPG.0000000000001642 file

Publishers page: <http://dx.doi.org/10.1097/MPG.0000000000001642>
<<http://dx.doi.org/10.1097/MPG.0000000000001642>>

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Title:

Anti-transglutaminase 6 autoantibody development in children with celiac disease
correlates with duration of gluten exposure

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Grant support: This work was funded by IRCCS Burlo Garofolo (Grant 03/15) Italy and Ryder Briggs Trust, UK

Word count of the manuscript body: 2189

Number of figures: 4

Conflict of interest: Daniel Aeschlimann serves as a scientific advisor/collaborator to Zedira (without financial incentives) but receives royalties from Zedira for patents. The other authors have no conflicts of interest to declare.

Author contributions: TN, MH, DA, and AV designed the study; TN, MH, and LDL analyzed the data and produced the first draft; GC, SM, and NS collected clinical data and follow up of all the patients enrolled in the study; LDL, DA, PA, SV, and FZ performed all the assays and critically analyzed the results.

Abstract

Objectives: Antibodies against transglutaminase 6 (anti-TG6) have been implicated in neurological manifestations in adult patients with genetic-gluten intolerance and it is unclear whether autoimmunity to TG6 develops following prolonged gluten exposure. We measured the anti-TG6 in children with celiac disease (CD) at the diagnosis time to establish a correlation between these autoantibodies and the duration of gluten exposure. We investigated a correlation between anti-TG6 and the presence of neurological disorders.

Methods: Anti-TG6 (IgA/IgG) were measured by ELISA in sera of children with biopsy-proven CD and of children suffering from gastrointestinal disorders. CD-patients positive for anti-TG6 were retested after 2 years of gluten-free diet (GFD).

Results: We analyzed the sera of 274 CD-children and of 121 controls. Anti-TG6 were detected in 68/274 (25%) CD-patients and in 19/121 (16%) controls, with significant difference between the two groups ($p=0.04$). None of the CD-patients and of the controls testing positive for anti-TG6 were suffering from neurological disorders. Eleven/18 (61%) CD-patients with other autoimmune diseases were positive for anti-TG6. In CD-patients a significant correlation between the gluten exposure before the CD-diagnosis and anti-TG6 concentration was found ($p=0.006$ for IgA; $p<0.0001$ for IgG). After GFD anti-TG6 concentrations were significantly reduced ($p<0.001$). No significant correlation was observed between anti-TG6 and anti-TG2 serum concentrations.

Conclusions: Anti-TG6 are more prevalent in children with untreated-CD in absence of overt neurological disorders. The synthesis of the anti-TG6 are related to a longer exposure to gluten before the CD diagnosis and the autoimmunity against TG6 is gluten dependent and disappeared during GFD.

Keywords: gluten free diet; neurological disorders; transglutaminase 6.

What is known/What is new

What is known:

- Celiac disease (CD) patients may have neurological manifestations triggered by gluten.
- Transglutaminase 6 (TG6) is expressed in neurons and is targeted by autoantibodies in patients with neurological manifestations.
- In the adult population anti-TG6 antibodies are a sensitive and specific marker of gluten ataxia and are gluten dependent.

What is new:

- The prevalence of anti-TG6 antibodies is 25% in untreated-CD children without neurological manifestations and is increased (61%) in untreated-CD children with other autoimmune diseases.
- In the pediatric population anti-TG6 antibodies correlate with the gluten exposure duration and disappear during gluten free diet.

Introduction

Celiac disease (CD) is a gluten dependent autoimmune disorder affecting genetically predisposed individuals bearing the HLA DQ2 or DQ8. CD related manifestations and symptoms are not confined to the gastrointestinal system and they ameliorate following strict gluten-free diet (GFD) ¹. Extra-intestinal manifestations include neurological, obstetric, hepatic, cardiac, dermatological (dermatitis herpetiformis), osteoporosis and juvenile chronic arthritis ²⁻⁷. Serum antibodies against the autoantigen transglutaminase type 2 (anti-TG2) are a hallmark of active CD and represent the first line serological testing for CD-diagnosis. In addition to TG2, two more transglutaminase enzymes, type 3 and 6, are involved in two distinct gluten-dependent disorders: dermatitis herpetiformis (DH) ⁸ and gluten-ataxia (GA) ⁹, respectively. Patients suffering from these two clinical conditions have serological evidence of these specific autoantibodies and some evidence for a causative role has been demonstrated ^{10,11}. Like anti-TG2 antibodies in CD, both the anti-TG3 and anti-TG6 antibodies are gluten dependent and their blood plasma titers decrease significantly in response to a GFD, with concomitant clinical amelioration for patients suffering from DH ¹² or GA ¹³. These specific autoantibodies can be useful in the diagnosis of such extra-intestinal manifestations. Untreated adult CD patients may also have anti-TG6 antibodies (range 14-38%) ^{9,13,14}. As yet it is not clear whether these patients are susceptible to the development of neurological dysfunction if they continue on a gluten-containing diet or if indeed they have subtle neurological dysfunction often missed due to lack of neurological evaluation. It is also unclear if these patients have anti-TG6 antibodies from childhood or whether they go on to produce them later on in life. Anti-TG6 antibodies amongst children with untreated CD have never been investigated, and only a single case of a child with CD and epilepsy in association with anti-TG6 ¹⁵ was reported. In this case, a dramatic response to the GFD was observed. In this study, we investigated the prevalence of the anti-TG6 antibodies in children with newly diagnosed CD. We compared the serum

concentration of anti-TG6 with the serum concentration of anti-TG2 at the time of diagnosis and following treatment with a GFD, and investigated a potential correlation with the duration of exposure to gluten containing diet before the diagnosis.

Patients and Experimental Procedures

Subjects and study design.

The study was performed retrospectively on sera collected from children with biopsy-proven CD diagnosed using the ESPGHAN criteria ¹⁶ at the Institute for Maternal and Child Health, IRCCS Burlo Garofolo (Trieste, Italy) between September 2011 and December 2013. Informed consent for the study was obtained from the parents of 274 children with biopsy-proven CD (178 F, 96 M;). In all of the CD patients intestinal biopsies the typical features of gluten sensitive enteropathy (villus atrophy, crypt hyperplasia and intraepithelial T lymphocytes density >25 T-cells/100 epithelial cells) were present.

As a control group we tested the serum samples from 121 children (69F, 52M; median age 11 years, range 1-16) suffering from various gastrointestinal disorders (44 with gastroenteritis, 17 with eosinophilic esophagitis, 13 with gastro-esophageal reflux, 41 with recurrent abdominal pain and 6 with foreign body ingestion) who had also undergone gastrointestinal endoscopy and biopsy.

Serum samples were stored at -30 C and all of the samples were thawed once, before being analyzed. All the subjects that tested positive for the anti-TG6 antibodies were retested after 24 months of GFD. Moreover, the CD related HLA DQ2/8 haplotypes were evaluated among all the CD patients and any control group subjects that tested positive for anti-TG6 by PCR. Positive anti-TG6 antibody results were correlated with the presence of ataxia or epilepsy by investigating patient records. Written informed consent was obtained from the parents of the children enrolled. The study was approved by the independent Ethical Committee of the Institute of Child Health IRCCS “Burlo Garofolo” (CE/V-04/2015).

Serology and HLA typing.

Serum IgA/IgG anti-TG2 were measured using an ELISA (Eurospital, Italy) following the manufacturer's instructions with normal values <7 U/I for both A and G immunoglobulin isotype. Serum IgA/IgG anti-TG6 were measured by means of an ELISA assay as previously described ¹⁷. A measurement >75 U/ml for IgA or >34 U/ml for IgG was considered positive.

The susceptibility alleles for CD were determined by PCR with allele specific primers identifying DQ2 and DQ8, using Eu-Gene-Risk kit (Eurospital, Italy).

The assays were performed by operators (LDL, DA, PA, SV, FZ) blinded to the subjects' clinical and hospital laboratory data.

Statistical analysis.

Correlation between the anti-TG6 antibody serum concentrations and age at time of CD diagnosis were evaluated by using nonparametric Spearman rank test, while the correlation between the anti-TG6 antibody and the anti-TG2 antibody serum concentrations were evaluated by using linear regression analysis. Statistical comparison between the serological data of the different groups was performed using the Fischer's exact test, and the sequential serum samples were also compared using Wilcoxon signed rank test. A value of $p < 0.05$ was considered significant.

Results

Serum autoantibody analysis.

Children with biopsy proven CD (274: 178F, 96M; median age 7 years, range 1-17) and children (121: 69F, 52M; median age 11 years, range 1-16) suffering from other gastrointestinal disorders (control group: 44 with gastritis, 17 with eosinophilic esophagitis, 13 with gastro-esophageal reflux, 41 with recurrent abdominal pain and 6 with foreign body ingestion) were investigated for TG2 and TG6 autoantibodies:

All of the CD patients tested positive for HLA DQ2 or DQ8 and anti-TG2 autoantibodies. Serum IgA anti-TG2 in this group ranged from 8 to 280 U/l (mean concentration 60 ± 52 U/l, normal values <7 U/l) and serum IgG anti-TG2 from 0 to 500 U/l (39 ± 62 U/l, normal values <9 U/l). In six patients with total IgA deficiency, IgG anti-TG2 titers above the threshold value were detected (mean concentration 82 ± 25 U/l). Anti-TG6 antibodies were found in 68/274 (25%) CD-patients (36 tested positive for IgG, 25 for IgA and 7 for both IgA and IgG) (Fig.1). There was a significant difference compared to controls ($p=0.04$). There was no mention of any neurological disorder in either patients positive or negative for anti-TG6 antibodies. This was based on retrospective evaluation of the patients' records. Eighteen out of 274 CD-patients were suffering from other autoimmune diseases (thyroiditis, type 1 diabetes, dermatitis herpetiformis) and 11 of them (61%) tested positive for anti-TG6 (9 for IgG and 2 for IgA).

No statistically significant correlation was observed between anti-TG6 and anti-TG2 serum antibody concentrations (Fig.2), in line with data from other patient cohorts ^{9,11}. There was a significant correlation between the duration of the gluten exposure before the CD-diagnosis and serum concentration of both IgA ($p=0.006$) and IgG ($p<0.0001$) anti-TG6 antibodies (Fig.3).

None of the controls tested positive for the anti-TG2 antibodies (mean concentration 1.5 ± 2 for IgA, 3.5 ± 2 for IgG U/l) and their intestinal biopsies did not demonstrate the CD-related abnormalities. Two out of 121 subjects belonging to subjects with gastrointestinal disorders (control group) were affected by IgA deficiency, and anti-TG6 IgA were thus measured in 119 patients. Anti-TG6 antibodies were found in 19/121 (16%) subjects in the control group (12 tested positive for IgG, 5 for IgA and 2 for both IgA and IgG) (Fig.1). Among the 19 subjects positive for anti-TG6 antibodies, 8 were suffering from recurrent abdominal pain (42%), 4 from gastritis (21%), 4 from gastro-esophageal reflux (21%) and 3 from eosinophilic esophagitis (16%). The CD-related HLA was present in 6 of 19 (31%,

all six tested positive for HLA DQ2). None of the controls were suffering from neurological disorders.

CD-patient follow-up after GFD.

Forty out of 68 (59%) children with CD that tested positive for anti-TG6 antibodies (18 tested positive for IgG, 17 for IgA and 5 for both IgA and IgG) were re-tested after 24 months of GFD. Thirty-eight out of 40 (95%) tested negative for anti-TG2 antibodies and 36/40 (90%) tested negative for anti-TG6 antibodies, with significant decrease in both IgA and IgG antibody serum concentration before and after GFD ($p < 0.001$) (Fig.4). Two out of the four subjects still positive for anti-TG6 did not follow gluten-free diet and were positive for anti-TG2 antibodies as well (IgA 40 and 65 U/I, IgG 39 and 25 U/I).

Discussion

In adult patients with CD presenting with neurological manifestations there is a significant difference of age at presentation when compared to adult patients with CD presenting to a gastroenterologist with the classic symptoms (53 vs 42 years)¹⁴. This therefore raises the question as to whether the respective underlying autoimmune responses develop at different consecutive phases in life, particularly in light of the fact that neurological problems are rarely seen in children with CD. Given that neurological manifestations are associated with an autoimmune response to TG6,^{2,9,13} we investigated whether children presenting with CD had circulating autoantibodies to TG6. Here, we show for the first time that such autoantibodies can be detected in young children at time of presentation with considerable frequency, suggesting that autoantibodies to different transglutaminase (TG) isozymes are developed simultaneously and not during different phases of life, i.e. as a late stage disease sequelae.

Interestingly, our data show a significantly higher prevalence/concentration of anti-TG6 antibodies among children with a longer exposure to gluten containing diet before the CD-

diagnosis. Moreover, these autoantibodies are gluten dependent, with elimination of anti-TG6 antibodies in 90% of patients following the GFD. This is in line with what is seen in pediatric CD patients who are positive for other organ-specific autoantibodies (e.g. anti-thyroid peroxidase antibodies, glutamic acid decarboxylase antibodies, anti-insulin antibodies) ¹⁸. Such autoantibodies may disappear following a GFD ^{18,19}, raising the possibility that in some instances gluten-dependent T cells can drive multiple immune responses and hence, that GFD could potentially prevent the development of neurological deficits or secondary autoimmune diseases such as type 1 diabetes and autoimmune thyroiditis in patients with genetic gluten intolerance.

We have found the prevalence of anti-TG6 antibodies to be 25%. This compares to a prevalence of 38% in adult patients of European Caucasian descent with newly diagnosed CD but from a different geographical region (UK) ¹⁴. The geographical selection (ethnicity) may well be an important factor as differences in the prevalence of anti-TG6 antibodies amongst adult patients with CD have been identified (38% in adult CD patients from the UK vs 14% in adult CD patients from Finland) ^{13,14}. This may be one possible explanation to variations in the prevalence of neurological manifestations amongst patients with gluten related disorders in different countries. The overall prevalence of anti-TG6 antibodies in our control group was 16% (specificity 84%) which is higher than what has been found in healthy controls (4%) ^{13,17}. This is likely to relate to the fact that most of these controls suffered from other gastrointestinal disorders associated with chronic inflammation with only six (with foreign body ingestion) being free from gastrointestinal disease, none of which were positive for anti-TG6 antibodies. It remains to be seen if some of these patients which carry the genetic gluten intolerance (HLA DQ2/8) may develop CD in the future.

Of interest is also our finding that 11/18 (61%) CD patients suffering from autoimmune diseases (thyroiditis, diabetes, dermatitis herpetiformis) tested positive for anti-TG6 antibodies, a significantly higher prevalence compared to the CD population as a whole.

This raises the possibility that the presence of anti-TG6 antibodies may be a marker of 'multiple' autoimmunity, perhaps signifying a different stage of the immune response.

Our finding of autoantibodies to different transglutaminase isozymes in children at presentation is consistent with the current view that B cells/plasma cells targeting TG6 originate independently from those targeting TG2 and not due to epitope spreading. This is supported by the fact that TG6 can generate gluten peptide-derivatives that drive both T and B cell responses ²⁰, anti-TG6 antibodies can be the only autoantibodies present in patients with genetic gluten intolerance ⁹, and analysis of clonal antibodies from celiac patients established that anti-TG antibodies are isozyme specific ²¹. Hence, not surprisingly, the serum concentration of anti-TG6 antibodies among untreated CD patients examined here showed no correlation to the presence of high anti-TG2 antibody serum concentration. This is consistent with the observation that anti-TG6 antibodies are present in up to 67% of adult CD patients who present with neurological dysfunction as opposed to 38% in adults with CD who present to gastroenterologists, yet the prevalence of TG2 antibodies in the 2 groups is similar (91-97%) ¹⁴. A similar observation can also be seen in patients with dermatitis herpetiformis where the immunological response targets TG3. Circulating anti-TG3 antibodies were found in 86% of patients with DH as opposed to 24% in patients with CD, while TG2 antibodies showed a similar prevalence in both DH (86%) and CD (92%) patients ¹². The role of circulating TG isozyme-specific autoantibodies in development of extraintestinal disease has indeed been demonstrated in animal models ^{10,11}.

The absence of neurological dysfunction amongst children with CD positive for anti-TG6 may be due to a number of factors. Firstly, our assessment of any neurological problems was retrospective and based just on the clinical records. None of the patients were evaluated by a neurologist or underwent full neurological examination and brain imaging to look for any evidence of neurological dysfunction. Secondly, neurological manifestations in

children with CD are said to be rare when compared to the adult population. An immune response targeting TG2 drives overt tissue destruction in the gut (due to antigen abundance) in short succession but this is not the case for an immune response targeting TG6. Hence, extraintestinal manifestation may be slow progressing or latent. It is also likely that neurological problems that are subclinical are missed for prolonged periods, hence the typically late diagnosis. In the case of TG6 autoantibodies, it is of course also possible that circulating autoantibodies by themselves are not sufficient to precipitate neurotoxicity as the CNS is shielded by the blood brain barrier but that an additional event is necessary for which there is an increased risk with age. The implication of this finding is that patients with the classic presentation of gastrointestinal symptoms are more likely to be diagnosed with CD than those with extra-intestinal manifestations. Extra-intestinal manifestations are a late phenomenon, perhaps associated with only mild gastrointestinal disease as a consequence of a different bias of the immune response. Evidence in support of this comes from the fact that the prevalence of DH in Finland has dramatically decreased since the introduction of CD screening programs ²².

It remains to be seen if patients with anti-TG6 antibodies and no enteropathy who do not go on a GFD may be at risk of developing neurological dysfunction after prolonged exposure to gluten. Careful follow up of those patients in the control group that are positive for anti-TG6 may be advised and of interest.

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Figure legend

Fig.1 Serum concentration of IgA and IgG anti-TG6 antibodies in the study groups. The cut-off limits for both IgA (>74 U/ml) and IgG (>34 U/ml) are indicated by the thin lines. CD: children with celiac disease.

Fig.2 Linear regression analysis regarding the relationship between the serum concentrations of anti-TG6 antibodies and of anti-TG2 antibodies in CD-patients. No statistically significant relationship between the serum antibody concentrations against the two antigens was found.

Fig.3 Correlation analysis of age (corresponding to time of gluten exposure) at CD-diagnosis with the IgA and the IgG anti-TG6 serum concentrations. A statistically significant relationship was found with both IgA and IgG anti-TG6.

Fig.4 CD patients tested positive for IgA/IgG anti-TG6 at the diagnosis time during the gluten containing diet (GCD) and after 24 months of gluten-free diet (GFD).

Fig. 1

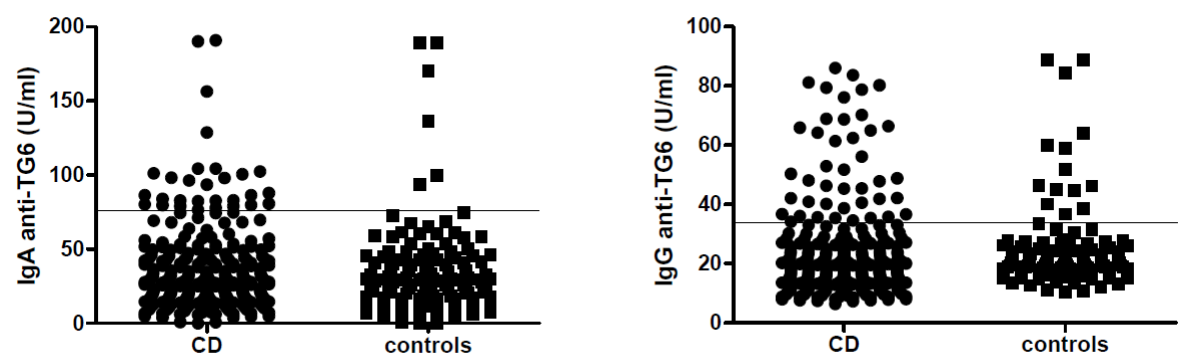


Fig. 2

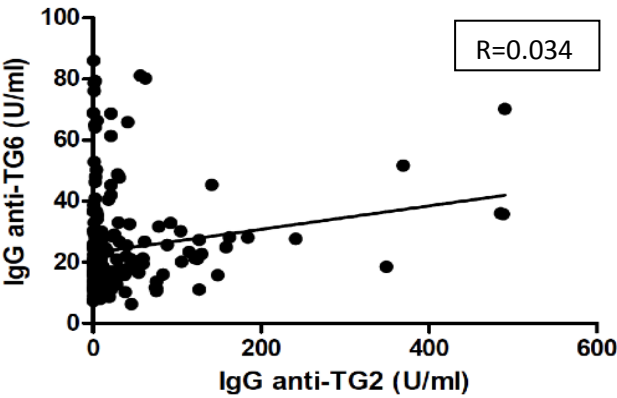
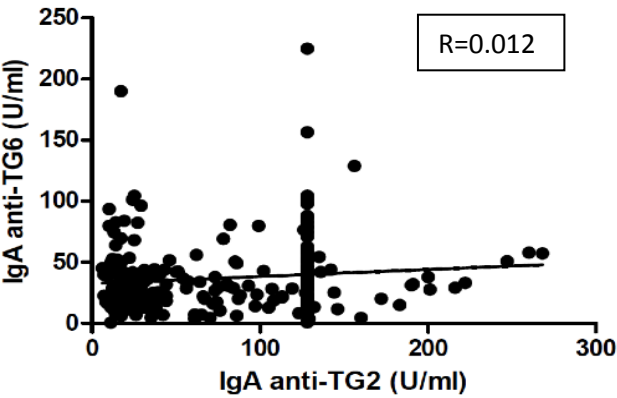


Fig. 3

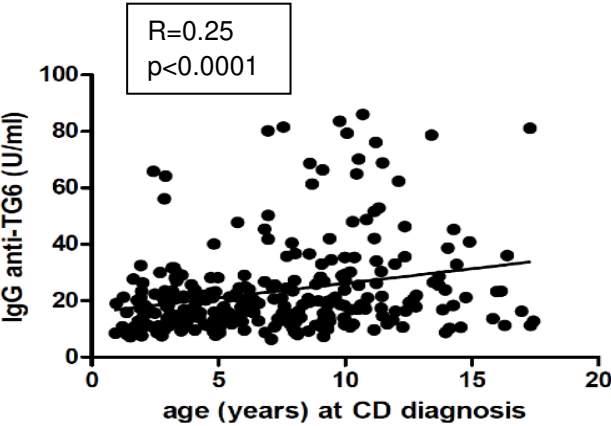
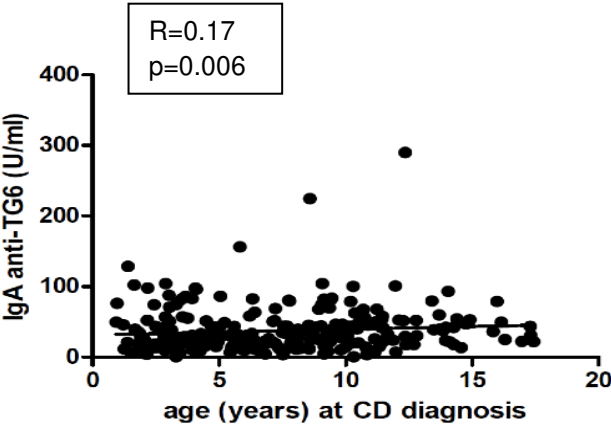


Fig. 4

